experience and familiality with materials may be required to determine stages accurately. It must be taken into consideration that the stages are artificially constructed by dividing a continuous change into steps. Naturally, there will often be discrepancies between actual embryos and illustrations. Stages of such embryos should be described such as, for instance, "early stage 20" or "late stage 21."

Some developmental events described in Table 1 may be less reliable than others as developmental criteria. It is relatively difficult to distinguish organ primordia in living embryos from stages 17 to 19, though they are observable more discretely in fixed or properly stained embryos. The earliest stage when the heart beat is recognizable in some embryos is stage 23 but the heart beat is observable in all embryos at stage 27. It is also difficult to

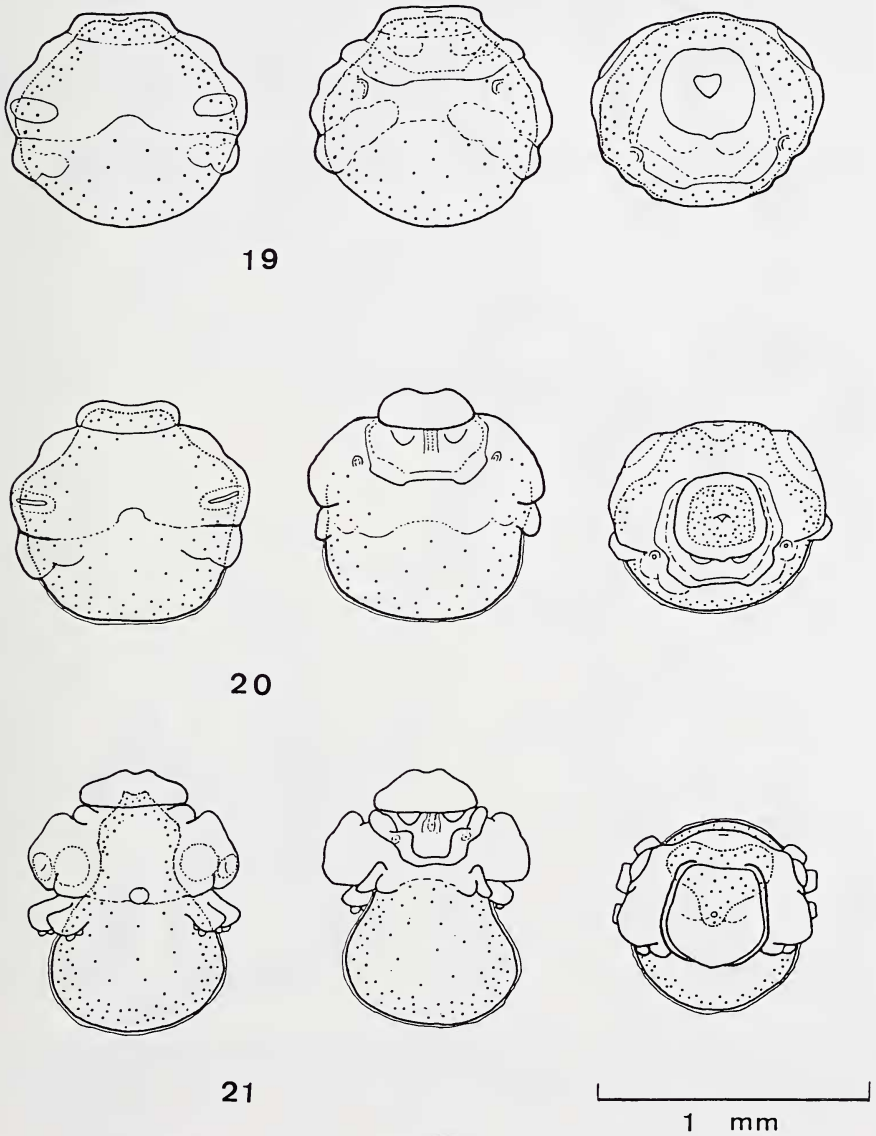

FIG. 4. Normal embryonic stages of *Idiosepius pygmaeus paradoxus*: Stages 19–21. The left, middle and right drawings in each stage are the dorsal, ventral and animal pole views, respectively.

determine the precise stage for the secondary cornea to cover the eye. The retina shows different colorations under different illuminations. Hatching occurs precociously at stage 28 if embryos are mechanically agitated. Thus, it is necessary to use several criteria to identify stages correctly.

The time data presented in Table 1 is useful only as an approximation. The speed of development varies among individuals or batches. Cleavage

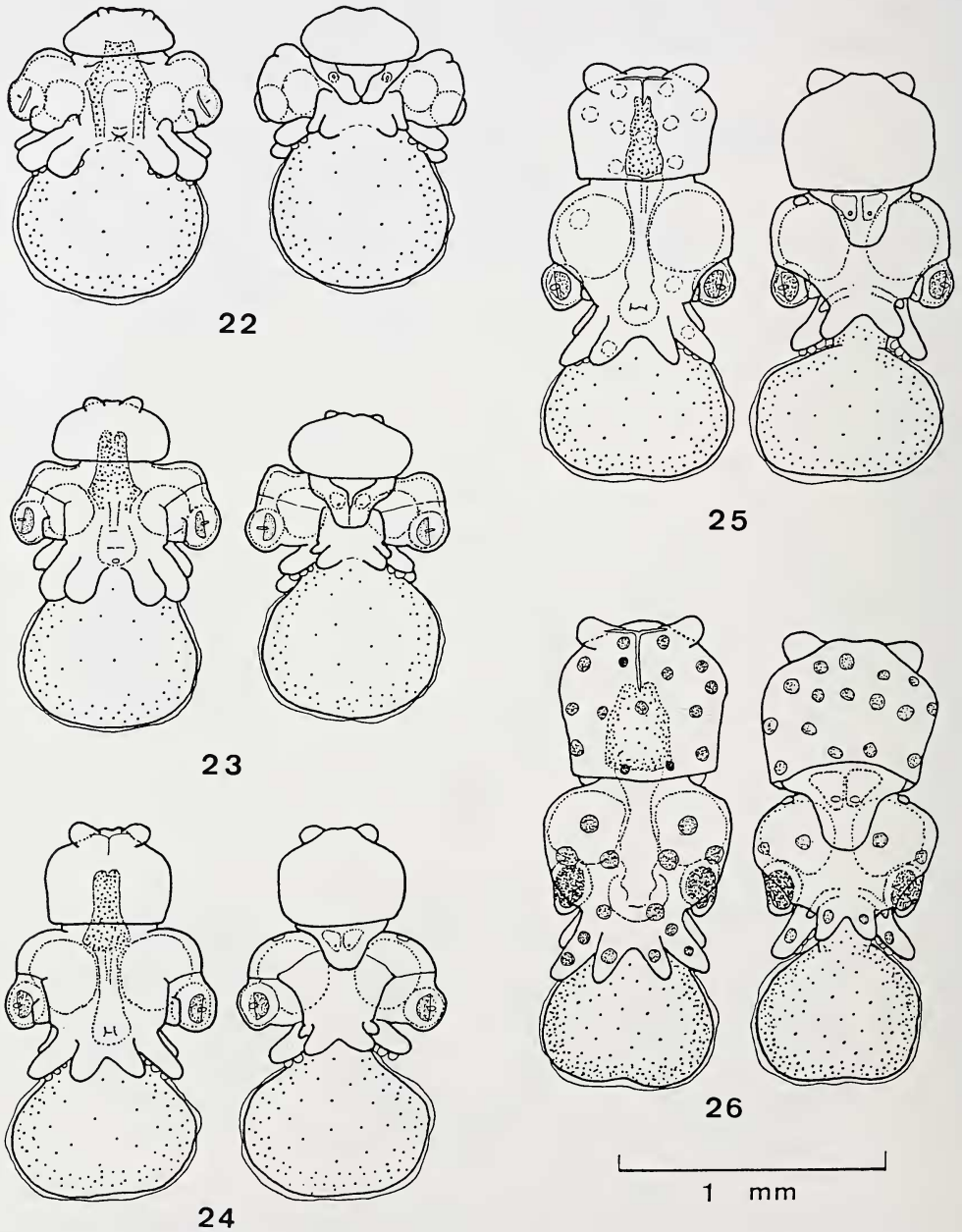

FIG. 5. Normal embryonic stages of *Idiosepius pygmaeus paradoxus*: Stages 22–26. The left and right drawings in each stage are the dorsal and ventral views, respectively.

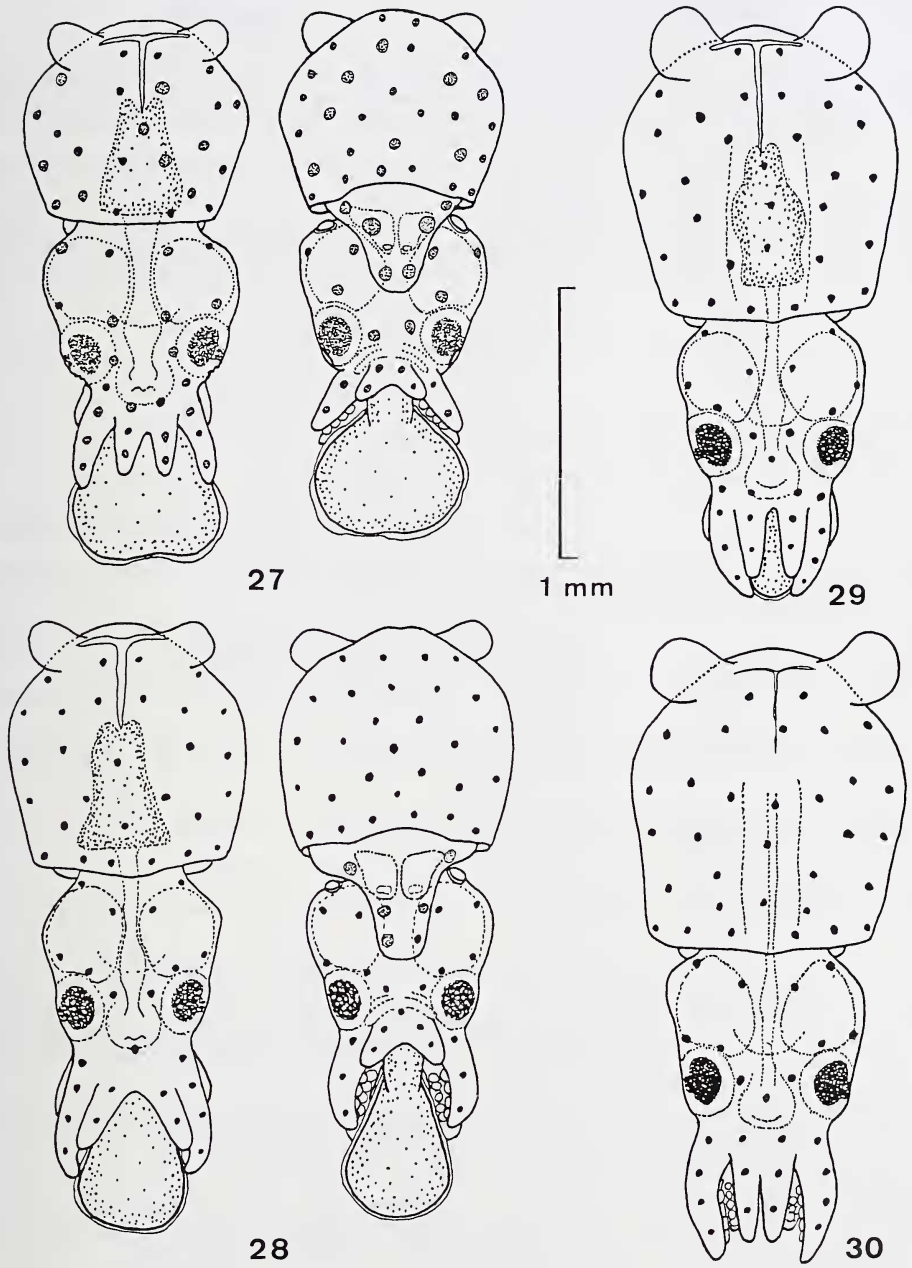

FIG. 6. Normal embryonic stages of *Idiosepius pygmaeus paradoxus*: Stages 27–30. The left and right drawings in stages 27 and 28 are the dorsal and ventral views, respectively. Only the dorsal views are shown in stages 29 and 30.

TABLE 1. Normal embryonic stages of the cuttlefish *Idiosepius pygmaeus paradoxus*

Stage No.	Time after oviposition (20±0.5°C)	Main developmental events
1		Precleavage stage. First polar body appears 45–60 min after oviposition (1a). Blastodisc becomes evident about 2 hr 30 min and second polar body appears about 3 hr 30 min after oviposition (1b).
2	5.5 hr	2 cell stage. First cleavage plane occurs along the plane of bilateral symmetry of the embryo.
3	7.0 hr	4 cell stage. Second cleavage plane occurs obliquely to the first one and the embryonic anterior (future dorsal) and posterior (future ventral) become evident. The polar bodies are present in the anterior.
4	8.5 hr	8 cell stage. Division is unequal.
5	10.0 hr	16 cell stage. Division is unequal.
6	12.0 hr	32 cell stage. Division is unequal and asynchronous.
7	14.0 hr	About 64 cell stage.
8	16.0 hr	Blastoderm is spreading by division of marginal cells.
9	20.0 hr	Blastoderm is indented in outline with increasing marginal cells.
10	24.0 hr	Blastoderm is decreasing in diameter by centripetal migration of marginal cells.
11	1 day 05 hr	Blastoderm is smooth in outline and smallest in diameter. A papilla of yolk is visible in the center of the blastoderm.
12	1 day 14 hr	Yolk papilla becomes flattened. Blastoderm has slightly increased in diameter.
13	2 day 00 hr	Epiboly begins. About 1/6 of the egg surface is covered by the blastoderm.
14	2 day 13 hr	About 1/4 of the eggs surface is cellulated.
15	3 day 01 hr	About 1/3 of the egg surface is cellulated.
16	3 day 14 hr	About 1/2 of the egg surface is cellulated.
17	4 day 03 hr	About 2/3 or more of the egg surface is cellulated. Major organ primordia appear as thickenings in the blastoderm.
18	4 day 16 hr	Most of the egg surface is cellulated. Primordia of the mantle, shell gland, mouth and arms are discernible but very faintly. Egg changes in shape from ovoidal (18a) to discoidal (18b) and begins to rotate very slowly.
19	5 day 05 hr	Cellulation is complete. Primordia of the gills, funnel folds and statocysts are visible faintly. Invagination of the eyes and shell gland has begun. Embryos rotate rapidly.
20	5 day 18 hr	Primordia of the mantle, funnel folds, gills and arms become discrete by elevation from the embryonic surface. Anus primordium is evident. Sucker primordia appear on some arms. Yolk sac begins weak pulsation.
21	6 day 07 hr	Embryonic body begins to stand up from the egg surface. Eye vesicles have just closed. Optic lobe primordia are visible. Sucker primordia have increased in number. Mantle begins to grow downward. Ventral funnel folds are bent toward the midline.

(continue)

Table 1. (continued)

Stage No.	Time after oviposition (20±0.5°C)	Main developmental events
22	7 day 00 hr	Fins are discernible with care. Funnel folds have just formed the siphon at the ventral proximities. Yolk sac is separated from the embryonic body by a constriction. Eye vesicle is spherical in shape. Lens is visible as a small dot. Retina is dish-shaped and very pale yellow.
23	7 day 18 hr	Mantle completely covers the gills. Fins are evident. Median margins of the ventral funnel folds are fusing. Retina is cup-shaped and reddish orange in color. Lens is evident as a refractile rod. Iris fold is being formed. Rotational movement gradually ends but pulsation of the yolk sac becomes strong. Heart beat begins in some embryos.
24	8 day 16 hr	Organ of Hoyle is discernible with care. Retina is reddish brown. Iris is prominent as a colored circle. Mantle completely covers the margin of the funnel.
25	9 day 16 hr	Chromatophores, very pale yellow in color, appear on the dorsal face of the mantle, at first, and of the head, later. Organ of Hoyle is discrete. Secondary cornea has just begun to cover the eye. Retina is dark brown. Olfactory organs are evident. Pulsation of the yolk sac weakens. Mantle sometimes contracts.
26	10 day 16 hr	Chromatophores, orange in color, appear on the whole embryonic surface. Secondary cornea is covering the eye. The mantle, head and yolk sac are approximately equal in size but the head is somewhat larger in width than the mantle. Embryos begin to move the fins, siphon and arms and often change position within the egg capsule.
27	12 day 06 hr	Secondary cornea has just covered the eye. Retina is reddish black. The mantle is slightly larger in width than the head.
28	13 day 22 hr	The mantle is prominently larger in size than the head. Yolk sac is decreasing in size. Chromatophores are usually contracted. Embryos move vigorously in the capsule.
29	15 day 22 hr	Yolk sac is very small or almost invisible but a mass of yolk remains in the embryonic body. Some embryos hatch.
30	18 day 05 hr	Yolk mass is completely lost. The remaining embryos hatch.

proceeds fairly synchronously within a batch but after epiboly, time variation between individuals gradually increases with stages; the fastest reaches stage 19 about 24 hr earlier than the latest. Developmental speed is also dependent on temperature and density of eggs. The duration from oviposition to hatching was about 12 days at 23°C and more than 1 month at 17°C. The embryos located in the central area of a broad egg mass always develop more slowly than those in its periphery and sometimes show abnormalities in shape, especially at late embryonic stages; the development may be susceptible to oxygen concentration.

The egg envelopes are omitted from the illustrations except one drawing in Figure 2. The perivitelline space between the egg and the vitelline membrane is narrow up to stage 17 but gradually increases in volume after stage 18. The first slight increase in volume at stage 18 may give the egg a room to change the shape and to rotate. Gomi *et al.* [7] have suggested in *Sepiella* that proteinaceous osmoactive substance produced by the embryo causes water intake into the perivitelline space.

There are many reports on the developmental process of the cephalopod [2, 8] but only a few are usable as normal tables of embryonic development. Naef [9] has presented the developmental series for many cephalopods in his extensive

monograph but cleavage is neglected in his stagings. Arnold [1] has divided the embryonic period of *Loligo pealii* into 30 stages, which do not always correspond to the present 30 stages. Yamamoto [10] has defined 40 stages in the embryonic development of *Sepiella japonica*. The embryo of *Sepiella japonica* is seemingly different from that of the present material; in the former the organogenetic period is longer and more stages are definable there than in the latter.

ACKNOWLEDGMENT

I would like to thank Messrs. W. Godo and M. Isozaki of this Marine Laboratory for their help in collecting the cuttlefish. This work is supported by a Grant-in-Aid from the Japanese Ministry of Education, Science and Culture (No. 60540463).

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Absence of Androgen Receptors in the Prostatic Glandular Epithelium Derived from Testicular Feminization Mutant (*Tfm*) Mice

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ABSTRACT—Urogenital sinus epithelium from male embryos of *Tfm* androgen receptor defective mice was combined with urogenital sinus mesenchyme from normal rat embryos and the recombinates were grown underneath the kidney capsule of male nude mice. The *Tfm* epithelium formed prostatic buds which developed into fully formed glands. A few recombinates also formed seminal vesicles and coagulating glands. A study of androgen receptors by steroid autoradiography showed that labelling was absent in the epithelium but present in the mesenchyme surrounding the epithelium. In the control recombinates in which epithelium from normal wild type mice was combined with the rat mesenchyme both epithelium and mesenchyme were androgen-labelled. A study of the fine structure of the *Tfm* epithelium and its enzyme activity showed that both approximate those seen in the control epithelium. The results suggest that epithelial androgen may not be required for the cytodifferentiation of the epithelium and that both bud formation and cytodifferentiation are induced by the androgen activated mesenchyme. The possibility that androgens are necessary for epithelial cell proliferation and the synthesis of specific prostatic proteins, is discussed.

INTRODUCTION

Prostatic glands develop from the urogenital sinus as epithelial buds projecting from the sinus epithelium into the surrounding mesenchyme under the influence of androgens [1]. Epithelial mesenchymal recombination experiments between androgen deficient (*Tfm* mutant) epithelium and wild type mesenchyme [2] suggested that the mesenchyme is a target for androgen and that epithelial buds are induced by androgen primed mesenchyme. This concept has been supported by steroid autoradiographic analysis which showed androgen-binding sites in the mesenchyme throughout all fetal stages [3]. In contrast androgen binding sites were absent in the fetal epithelium but appeared after birth coinciding with the cytodifferentiation of the postnatal epithelium [4, 5].

This paper attempts to relate the presence of androgen to the development and cytodifferentiation of the mouse prostate gland, and also to determine whether the urogenital sinus mesenchyme will induce androgen receptors in the androgen receptor deficient *Tfm* epithelium. For this purpose urogenital sinus epithelium from the *Tfm* mouse fetuses was combined with urogenital sinus mesenchyme from normal rat fetuses and the recombinates grown under the kidney capsule of normal host mice, for up to 8 weeks. The fine structure of its epithelium and the enzyme activity of epithelium and mesenchyme such as alkaline phosphatase and nonspecific esterase activity were determined. The uptake of labelled androgen by the mesenchyme and epithelium was analysed by steroid autoradiography and the results correlated

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with the degree of cytodifferentiation and enzyme activity.

MATERIALS AND METHODS

Animals

Mice carrying the *Tfm*-mutation gene and wild-type mice were kindly provided by Dr. Mary F. Lyon (MRC, Harwell, Oxon) and bred in inbreeding in our laboratory. Mutant male (X^{Tfm}/Y) embryos were obtained by mating X^{Tfm}/X^{+} females with wild males (X^{+}/Y). Wistar-Imamichi rats were purchased from Imamichi Institute for Animal Reproduction, Oomiya, Japan. The animals were mated during the night and copulation was confirmed by the presence of spermatozoa in the vaginal smears on the next morning. The conceptus was considered to be 0.5-day old at 12:00 of this day. Male fetuses of 15.5- and 16.5-days were obtained from mother mice and rats, respectively. Post-natal Wistar-Imamichi rats were also used for control.

Identification of Tfm-mutant fetuses

Fragments of pectoral skin of all male mouse fetuses used for the study were examined histologically. Mammary rudiments regressed and no hair follicles appeared in 15.5-day wild-type (X^{+}/Y) fetuses, while both rudiments were developed in mutant (X^{Tfm}/Y) ones.

Chemicals

Cold testosterone (Koch-Light Laboratories, Colnbrook) and [1, 2, 6, 7-^{3}H] testosterone (83.4 Ci/mmol; Amersham International PLC, Bucks) dissolved in propylene glycol and this stock solution was further diluted with medium 199 (Earle's salts, GIBCO Laboratories, Grand Island, NY, U.S.A.). Collagenase (Worthington Biochemical Corporation, Freehold, NJ, U.S.A.; Code CLS 149 U/mg) was dissolved in Tyrode's solution.

Separation of epithelium and mesenchyme

Urogenital sinuses of fetal male mice and rats were treated with 0.06% collagenase solution (Worthington Biochemical Corp., Code CLS) for 40 min at 37°C, and the epithelium and mesenchyme were separated carefully by fine two pairs of forceps. The tissues were then thoroughly washed in Tyrode's solution supplemented with 50% fetal bovine serum.

Recombination of epithelium and mesenchyme and cultivation of recombinates

Epithelia isolated from *Tfm* or wild-type fetuses were recombined with mesenchymes isolated from normal rat fetuses. Recombinates were cultured *in vitro* for one day by a modified Trowell technique [6, 7] in Earle's Medium 199 supplemented with 10% fetal bovine serum and 0.01 μg/ml cold testosterone. The recombinates were then grafted beneath the kidney capsule of male athymic ICR nude mice (Charles River Japan Inc., Atsugi, Japan), and cultured for 4, 6 and 8 weeks.

Histology and ultrastructural study

The grafts were removed from the kidney capsule in Tyrode's sloution supplemented with 10% fetal bovine serum and were divided into fragments approximately 1 mm thick. Some fragments were fixed in Bouin's fluid and the sections were stained with haematoxylin-eosin. Some fragments were fixed in a modified Karnovsky's fixative [8] at 4°C for 3 hr and post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.5) at 4°C for 2 hr, and carried through a graded series of alcohols, and embedded in epoxy resin according to the method of Luft [9]. Ultra-thin sections were cut on a Porter-Blum MT2 microtome, and stained with uranyl acetate and lead citrate. They were examined with a JEOL 100 CX electron microscope.

Enzyme histochemistry

Some other fragments of the grafts were fixed in ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2–7.4) for 2 hr, washed overnight in several changes of 5% sucrose at 4°C and frozen in isopentan (−190°C) chilled with liquid nitrogen. Sections, 5 μm thick, were cut in a cryostat (Cryocut II; American Optical, Buffalo, NY, U.S.A.) at about −25°C. Alkaline phosphatase activities were assessed by tetrazolium reaction (modified after McGadey, [10]) with 5-bromo-4-chloro-3-indoxyl-phosphate as the substrate and tetranitro-blue-tetrazolium as the coupling dye at pH 9.2–

9.4. Nonspecific esterase activities were assessed at pH 5.0 by an azo-coupling method [11] with 1-naphtyl acetate as the substrate and hexazotized para-rosaniline as the coupling dye.

Labelling procedure and autoradiography

Steroid autoradiography for locating androgen-binding sites was carried out according to the method of Stumpf and Sar [12]. The details of the labelling procedure and autoradiographic techniques were described in the previous paper [4]. The hosts were castrated 4-days before labelling to reduce the level of endogenous androgens. Tissue recombinates were divided into small fragments approximately 1–2 mm thick. They were incubated in Medium 199 supplemented with 10% fetal bovine serum and 2.5 μCi/ml [1, 2, 6, 7-^{3}H]testosterone. The incubation was carried out at 37°C in a gas phase of 50% O_2 and 5% CO_2.

After 15 hr incubation the tissues were washed with Tyrode's solution for 3 hr and were rapidly frozen in liquid nitrogen. Five-μm frozen sections, cut in a cryostat, were thaw-mounted onto the emulsion-coated slides and exposed for 2–3 weeks at 4°C.

To assess the specificity of the binding of [^{3}H]testosterone, we added a 400-fold excess of unlabelled testosterone to the medium containing [^{3}H]testosterone.

Identification of tissues

Sections cut in a cryostat were stained with Hoechst dye #33258 (Calbiochem-Behring, La Jolla, CA, U.S.A.). By this staining, we can distinguish mouse nuclei from rat ones. Mouse nuclei contain several small discrete intranuclear fluorescent bodies, absent in rat nuclei.

RESULTS

Morphology of prostate glands induced in recombinates

Histological examination of the recombinates showed that most of them developed prostate glands. In recombinates of wild type epithelium and rat mesenchyme the acini had a wide lumen lined with columnar secretory epithelium which exuded much secretory matter. The lamina propria surrounding the epithelium was poor in cells and fibres (Fig. 1). Recombinates with *Tfm* epithelium and rat mesenchyme also formed glands but their tubules were narrower and the epithelium was lower with a reduction of the supranuclear cytoplasmic area. In contrast to glands induced from normal epithelium the connective tissue surrounding the epithelium was increased significantly (Fig. 2). With the increase of the culture period to 6–8 weeks the diameter of the tubules increased and there was some secretory matter. Some recombinates also formed seminal vesicles, and urethral and coagulating glands (Table 1). Hoechst staining confirmed that the epithelium in the recombinates consisted of mouse cells only (Fig. 3).

Ultrastructure of induced glands in the recombinates

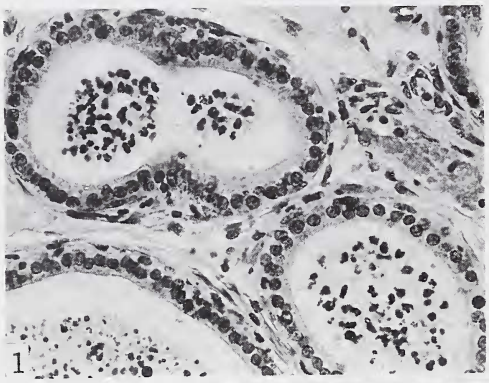
In the recombinates of *Tfm* epithelium with rat mesenchyme after 4 weeks growth microvilli developed at the apical surface of the epithelial cells, which also possessed a well developed rough surfaced endoplasmic reticulum and Golgi apparatus. Many secretory granules were seen in the apical cytoplasm (Fig. 4). These features were similar to those of the ventral prostate of the wild-type mouse at 4 weeks of age (Fig. 5).

Enzyme histochemistry

Alkaline phosphatase and nonspecific esterase activities were examined histochemically (Table 2). Recombinates of the sinus epithelium of *Tfm* mice and the sinus mesenchyme of normal rats showed similar activities to those of the control explants; the alkaline phosphatase activity was localized only in the lamina propria surrounding the epithelium which showed no activity (Figs. 6 and 7). In contrast, the nonspecific esterase activity appeared mainly in the cytoplasm of the epithelium (Figs. 8 and 9). These features are the same as those seen in normal prostate glands of the same age or in glands in the control explants.

Androgen receptors in the explants

We examined, then, whether androgen receptors were induced in the prostatic epithelium de-



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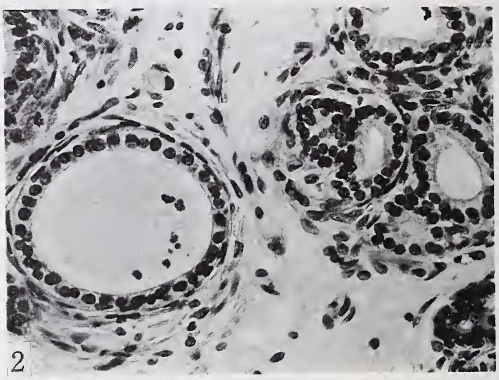

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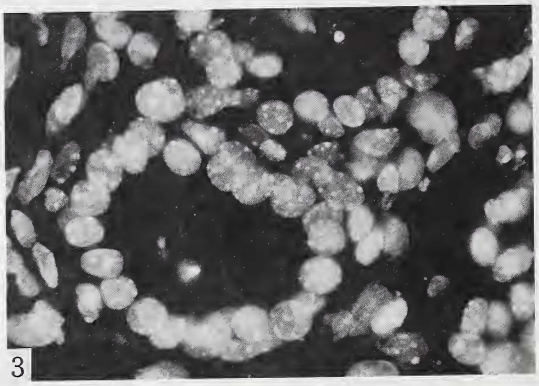

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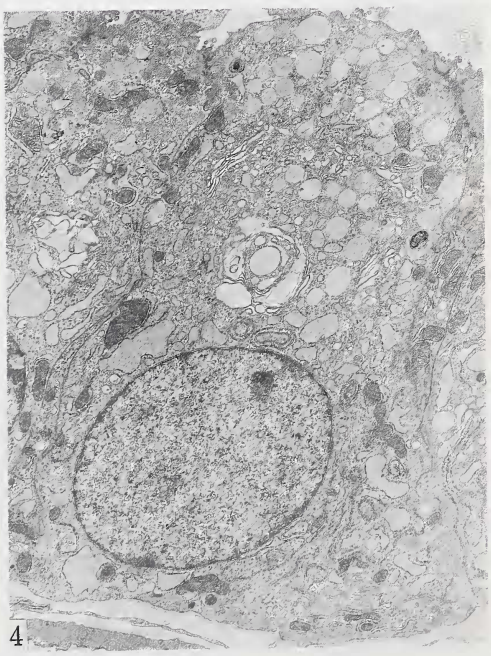

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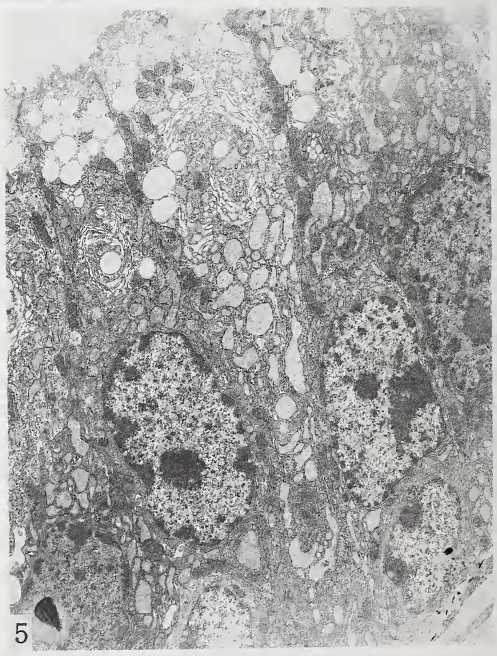

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